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Concentration and characterization of the emetic principle present in barley infected with Gibberella saubinetii (Mont) Sacc

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CONCENTRATION AND CHARACTERIZATION OF THE EMETIC PRINCIPLE PRESENT IN BARLEY INFECTED WITH GIBBERELLA SAUBINETII (Mont.) Sacc.

by

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INTRODUCTION

The toxicity of barley infected with Gibberella saubinetii (Mont.) Sacc. to man and certain animals has been well established. The first reports concerned the toxicity of flour made from the diseased grain. Later, a number of feeding experiments demonstrated the toxicity of scabby barley to animals, especially those with simple stomachs.

Further complaints will undoubtedly arise from utilizers of barley because epiphytotics of Gibberella saubinetii on barley are not uncommon and no barley has been developed which is immune to this fungus. Even though an immune variety of barley were developed, the permanence of this immunity is questionable because of the genetic variability existing within this pathogen.

With such conditions existing as outlined above, scabby barley may well be of as much concern to man in the future as it has been in the past. Although the toxicity of such grain toward certain animals has been well established, the information published concerning the nature of the emetic principle is limited and conflicting. Therefore, the present investigation was undertaken to learn something more of the characteristics of the emetic principle.

REVIEW OF LITERATURE

The first evidence indicating the toxicity of scabby barley as feed for animals was published by Eriksson in 1912. He reported that seeds affected with forms of Fusarium avenaceum are poisonous and produce dizziness and headache in man and beast. Since his report, studies have been made upon the effects of this toxin on various animals and its chemical composition. The more pertinent papers are reviewed.

Naumov (1916) reported that Fusarium roseum and F. subulatum attacked rye, wheat, oats and barley causing the grain to have toxic properties when it was used as a source of flour for bread. He designated bread made from such flour as "intoxicating bread". Dounin (1926) has designated such bread as "inebriant bread". He observed the extraordinary prevalence of Fusarium roseum on rye in Russia during the summer of 1923. According to him, people who ate bread made from this rye suffered from weakness, vertigo, headache, nausea and vomition.

Several investigators have established the fact that barley naturally infected with Gibberella saubinetii is toxic to swine. Such symptoms as vomiting, intoxication and emaciation have been reported. Experiments reported by Beller and Wedemann (1929), Oppermann and Doenecke (1929), Popp (1930), Mains, Vestal and Curtis (1930), Mundkur and Cochran (1930), Dickson, Link, Roche and Dickson (1930), Roche, Bohstedt and Dickson (1930), Mundkur (1934) and Christensen and Kernkamp (1936) indicate that swine are materially effected after having eaten grain infected with G. saubinetii.

The majority of investigators have indicated that scabby barley may be utilized as a feed for poultry with no apparent ill effects, among these are Mains, Vestal and Curtis (1930) and Roche, Bohstedt and Dickson (1930). Mundkur (1934) also reported no serious effects but noticed that rations containing infected barley were not relished. A comparison of the feeding value of rations containing relatively large quantities, respectively, of scabby barley, normal barley and yellow corn was made by Titus and Godfrey (1934). Their work indicated that rations containing slightly, moderately or very badly scabbed barley gave essentially the same results as normal barley so far as maintenance of live wieght, egg production and economy of egg production were concerned. Mundkur and Cochran (1930) fed mature chickens scabby barley and reported no ill effects, while chickens two weeks old showed a loss in weight and developed a rough plumage. Popp (1930) has observed that scabby barley produced illness in poultry.

Guinea pigs do not relish an exclusive infected barley diet as indicated by the work of Mundkur and Cochran (1930). When fed on an equal mixture of scabby barley and mash, they lost weight but did not develop any symptoms. The same results were also reported by Mundkur in 1934. Mains, Vestal and Curtis (1930) have obtained somewhat similar results with their guinea pig experiments.

Available evidence indicates that ruminants are not affected from eating scabby barley. Roche, Bohstedt and Dickson (1930) reported that cattle and sheep made good gains on scabby barley. Mains, Vestal and Curtis (1930), in their feeding experiments with scabby barley, state that cows ate the feed readily and no effect was noticed on weight, physical

condition, milk production or milk quality as compared with the previous ten days.

Other animals which have been subjected to feeding trials with scabby barley are dogs and rats. Roche, Bohstedt and Dickson (1930) reported that the former are sensitive to scabby barley. The only reported work with rats is that of Mains, Vestal and Curtis (1930). Their experiments indicated that rats were more susceptible than swine and in some cases death occurred. In cases where the rats died, an autopsy revealed no symptoms of disease.

Very little information has been published concerning the characteristics of the emetic principle present in grain infected with Gibberella saubinetii and related Fusarium species. Pomaskii (1915) was the first to offer any evidence as to the nature of the toxin. According to him, Fusarium roseum and F. subulatum act similarly on rye by dissolving starch and decomposing albumins. Other changes were noted in the pentosan, fiber and fat. There was a decrease in the iodine number and an increase in the acid number. Among the products of the decomposition of the albumins was a toxin which was thought to be a nitrogenous glucoside.

A later paper concerning the changes in the chemical composition of rye infected with Fusarium roseum and F. subulatum was reported by Pomaskii in 1916. Both species were able to decompose starch and protein. The products of the decomposition of protein were albumose, peptones, amino acids, organic bases, ammonia and a toxin, probably a nitrogenous glucoside. Changes were also noted in the pentosans, cellulose and fat.

According to Naumov (1916), if grain infected with Fusarium roseum

and F. subulatum is stored under ordinary conditions for a period of three years, the mycelium loses its viability. Shands (1937) states that Gibberella saubinetii remained viable in infected grain for at least 27 months. The work of Shands further indicates there is no correlation between the viability of the mycelium and the presence of the emetic principle. His tests with swine showed that the toxin remained highly active at 56 months after the infected barley was harvested. At this time, the fungus had been nonviable for several months.

A few attempts have been made to produce the emetic principle under various artificial conditions. Miessner and Schoop (1929) administered large quantities of artificial liquid cultures of Fusarium roseum to swine with a stomach tube in an attempt to produce emesis, but only excess feces deposition was observed. They also dispensed cultures of F. roseum in the feed of swine and again obtained negative results with respect to emesis. According to Popp (1930), pure cultures of Gibberella saubinetii were not poisonous to swine. Unobjectionable barley artificially inoculated with G. saubinetii did not develop the toxic substance. Although Davis (1938) did not test his material on swine, he has shown that a substance was present in the culture filtrate of G. saubinetii which was very effective in retarding the growth of this fungus. On the basis of the above investigations, it is not likely that this substance is of the same nature as the emetic principle produced in naturally infected barley.

As stated by Christensen and Kernkamp (1936), the chemical formula for the toxic substance has not been determined, but a few suggestions as to its chemical nature have been published. A chemical investigation of

the American barley by Danckwortt (1929) indicated that it could not be a question of poisoning with heavy metal salts in large quantities. Only traces of hydrocyanic acid were detected and the examination for alkaloids proved negative. No appreciable increase in the amount of ammonia, volatile amine bases or hydrogen sulphide was observed. Popp (1930) and Schroeter and Strassberger (1931) also made chemical investigations of the toxic American barley. Popp's investigation disclosed variation in the composition of the barley. In addition to the carbohydrates, the protein compounds had especially been decomposed to toxalbumins or similar toxic nitrogen compounds which brought about the sickness to the swine. The results of Schroeter and Strassberger lead them to believe that choline or easily hydrolyzable choline fatty acid esters produced the injurious action. Dickson, Link, Roche and Dickson (1930) succeeded in freeing the aqueous extract of scabby barley from protein, polysaccharides and those nitrogenous substances precipitable with tannic acid. They concluded the active substance or materials were associated with the fractions containing glucoside or basic nitrogen compounds.

Beller and Wedemann (1929) made an extensive investigation of the American barley and a detailed discussion of their work deserves consideration in this review. They tested the solubility of the toxin in water by shaking one kilogram samples of the American barley with one and a half liters of tap water for 14 to 20 hours. When 420 to 750 milliliters of this extract was administered to swine of 50 to 80 pounds, emesis was produced approximately 15 minutes later. No apparent after effects were noticeable and the experimental animals soon ate their regular feed. A

second extract from the same barley was tested for the presence of the toxic principle, but tests showed the principal quantity of the toxin had been removed from the barley during the first extraction.

The aqueous extracts were acidic and appeared turbid and coffee or cocoa colored. Inorganic material, starch, sugar and traces of alcohol were present. To be sure the small amount of alcohol present did not induce emesis, Beller and Wedemann administered one, three, five and seven percent alcoholic solutions to swine. No reaction was evident in any case.

Tests were made to determine the location of the toxic substance by removing the glumes and extracting these and the remainder of the kernels separately. Only the aqueous extracts from the glumes proved toxic. This led Beller and Wedemann to conclude that the toxin was present in the glumes.

Beller and Wedemann (1929) removed the toxin from the diseased grain by placing the barley in boiling water for one hour. Extracting with a five percent solution of hydrochloric acid also proved successful in removing the emetic principle. When the barley was extracted in a weak soda solution, the extract was not toxic. In the latter case, they concluded the toxin was removed from the grain but had undergone a chemical transformation since it was no longer capable of producing emesis. Christensen and Kernkamp (1936) succeeded in reducing the toxic effect of aqueous extracts of scabby barley by adding glucose, starch, flour or milk.

The evidence obtained by Beller and Wedemann concerning the possibility of producing the emetic principle under artificial conditions confirms the investigations previously reviewed. They obtained no injurious symp-

toms in swine by administering pure cultures of Gibberella saubinetii or aqueous extracts of pure cultures. Aqueous extracts from healthy barley which had been inoculated with the organism and incubated 11 to 30 days were noninjurious. A further test was made with a suspension of G. saubinetii spores and negative results were again obtained.

Beller and Wedemann did not report extensive experiments to determine the exact chemical nature of the toxin but concluded it was organic. They were certain, however, that swine receiving American barley in their rations over a period of several weeks showed no visible injuries. For the utilization of the toxic barley as feed for swine, they suggested the following practices: (1) by removing the glumes and (2) by extracting one hour in boiling water with subsequent washing or neutralization of the toxic substance with soda.

MATERIAL AND METHODS

During the summer of 1938, a number of barley fields in the vicinity of Ames, Iowa, were observed from the latter part of June until harvest. These observations disclosed a general epiphytotic of scabby barley. One field of Ioglos barley, located at the Iowa State College Agricultural Engineering Department Farm, was heavily infected with Gibberella saubinetii. Preliminary tests with this barley, previous to harvesting, indicated that it contained the emetic principle. In order to obtain more heavily infected barley than the ordinary field run, arrangements were made with the Agricultural Engineering Department to obtain the screenings. Screenings weighing 10, 15, 24, 27 and 32 pounds per bushel were used throughout the investigation. The screenings weighing 10 and 15 pounds per bushel contained much plant refuse and recleaning was necessary. Platings and germination tests indicated a negative correlation between the weight of the barley and the amount of infection. Although the bushel weights differed considerably, a small difference was noted in the amount of infection. Table I shows the average values for the respective bushel weights.

Table I

Percent infection with Gibberella saubinetii

	Bushel weights				
	10	15	24	27	32
Percent infection	95	94	90	90	87

A review of the literature indicated that swine were very susceptible to the emetic principle. For this reason, and because of the fact that constant dosage might reveal some injurious effects, swine were chosen as the assay animals to test for the presence of the toxin in various extracts and concentrates. During the course of the investigation, 16 swine of various breeds were administered the extracts and concentrates by using a stomach tube. Small pigs were generally more susceptible to the emetic principle and much variation among the 16 pigs, in the amount of emesis produced from equal amounts of scabby barley, was quite apparent. The swine were obtained when weighing not less than 25 pounds or over 50 pounds and retained until reaching a weight of approximately 150 pounds. Although the heavier swine were still susceptible to the emetic principle, they became more difficult to handle and were discarded for that reason. In some cases the swine were fasted approximately 12 hours before any material to be tested was dispensed. Since swine not fasted reacted equally well to the extracts and concentrates, fasting was not regarded as an important factor. After administering the test solution, the swine were observed until emesis had subsided. In only a few cases was it necessary to observe the assay animals more than one hour.

In addition to swine, pigeons were also tested for use as assay animals. To administer the toxin, a small rubber tube was introduced orally until reaching the crop. Even though pigeons were more convenient to handle and regurgitated after the dispensation of the toxic material, they were abandoned because of their failure to react within a certain time. In some cases regurgitation succeeded dispensation of the

test solution by only a few minutes and in other cases it was a matter of hours.

Aqueous extractions, as well as extractions with other solvents, were made from the whole grain. Other solvents tried for the first extraction, but not chosen for the standard procedure developed, were methyl alcohol, ethyl alcohol and ether. The aqueous extracts were made by placing a weighed quantity of infected barley in beakers and approximately twice the weight of distilled water added. The beakers were placed at various temperatures ranging from 7.2°C. to 100°C. and allowed to remain from a minimum of one hour to a maximum of two hundred sixteen hours. Twelve hours extraction with water at a temperature of 92°C. was chosen as a part of the regular procedure. The aqueous extracts were then decanted and the barley washed once with a small amount of distilled water. The washings were added to the first aqueous extracts.

Soxhlet extractors were employed when methyl alcohol, ethyl alcohol and ether were used as solvents. Methyl and ethyl alcohols proved to be efficient solvents for extracting the toxin from the whole grain but were abandoned because of the added material they extracted which made further purification of the toxin more laborious and difficult. Ether failed to remove the emetic principle from the whole grain.

After establishing the most satisfactory method for extracting the emetic principle from the infected whole grain, further methods were employed for the purification of the toxin. Vacuum, steam and fractional distillations were attempted before the aqueous extracts were concentrated over a water bath followed by the use of differential solvents. Chemical

properties of the emetic principle were noted during the purification procedure as well as during the attempts to inactivate it. The final extraction was made with ether in the specially constructed extractor shown in figure 1.

The stability of aqueous solutions of the emetic principle toward heat and to changes in the hydrogen ion concentration of the medium in which it was dissolved was studied during the development of the separation procedure.

Solubility behavior, elementary analysis, physical examination, homologous tests and an analysis for certain elements were carried out with the yellow syrupy concentrate. Attempts were made to crystallize, to isolate as a hydrochloride salt and to prepare various derivatives of the yellow syrupy concentrate.

After obtaining doubtful alkaloidal tests with the yellow syrupy concentrate, the latter was dissolved in a chloroform-ether mixture and extracted with a two percent hydrochloric acid solution in order to bring down any complex organic bases. The acid soluble portion was tested with several alkaloidal reagents.

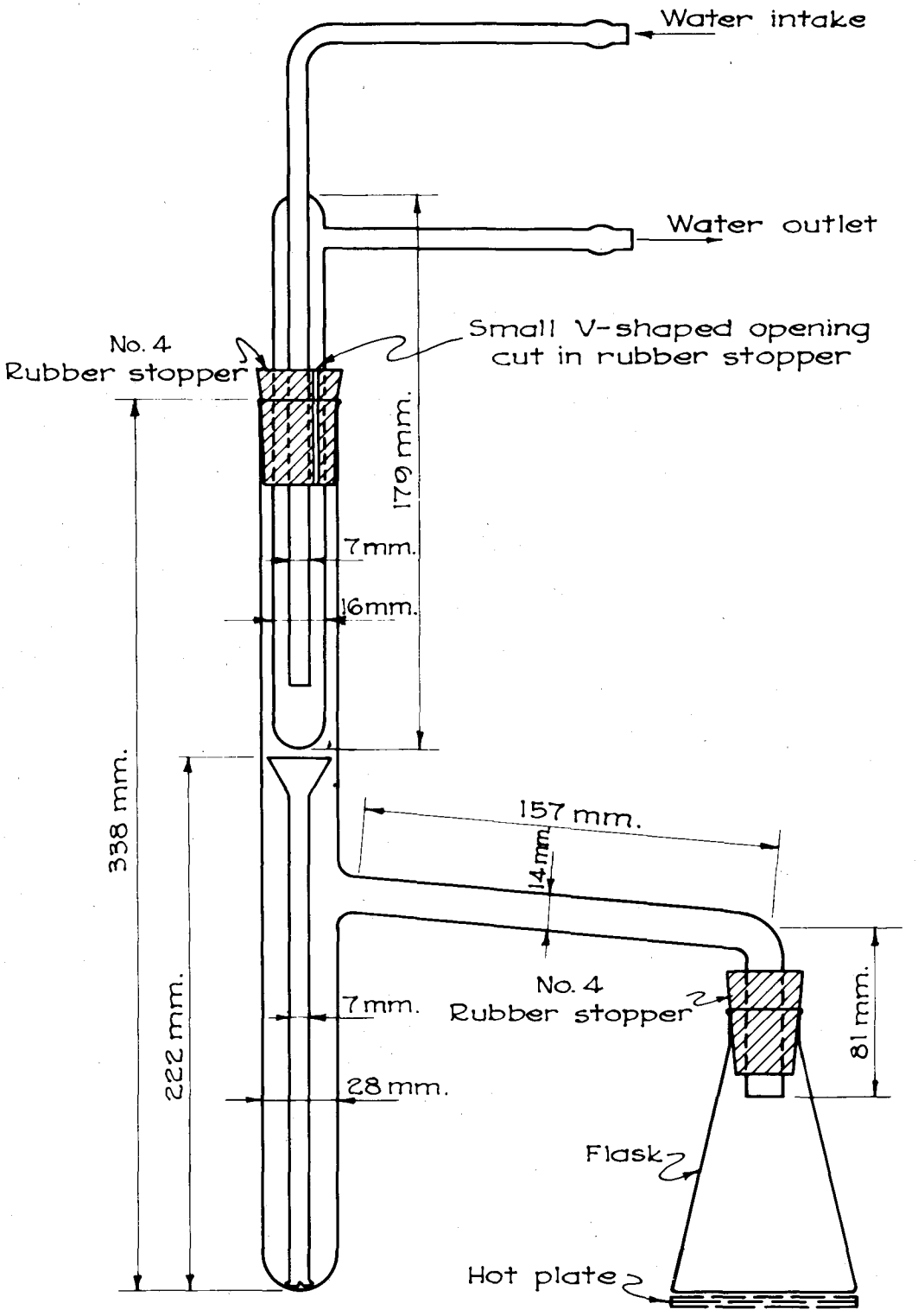

Figure 1. Extractor with condenser used for extracting small volumes.

The following scheme represents the procedure followed during the concentration of the emetic principle.

A weighed quantity of infected whole barley placed in beakers with approximately twice the weight of distilled water and heated at 92°C. for 12 hours. The extract decanted off and the barley washed once with a small volume of distilled water.

↓

Aqueous extract evaporated to nearly dryness on water bath. Brown residue dissolved in warm methyl alcohol and filtered with suction.

Barley discarded.

↓

Methyl alcohol soluble portion evaporated on steam bath to a small volume. Distilled water added and evaporation of methyl alcohol completed. Brown residue dissolved in small volume of dilute NaOH solution and placed in extractor shown in figure 1. Extracted 24 hours with ether.

Insoluble brown residue discarded.

↓

Ether soluble portion. Add anhydrous Na_2SO_4, dry and pore ether soluble portion on watch glass. Evaporate ether off.

Ether insoluble portion discarded.

↓

Yellow syrupy concentrate.

EXPERIMENTAL

Toxicity of the Aqueous Extracts

Previous investigations have demonstrated the fact that the emetic principle present in barley infected with Gibberella saubinetii is soluble in water. Since aqueous extracts were a part of the regular procedure, a number were tested for their potency when different quantities of barley were extracted at different temperatures for various lengths of time. Table II summarizes these data.

Table II

Toxicity of the Aqueous Extracts

Pig Number	Grams of scabby barley extracted	Weight of barley per bushel, pounds	Temperature extracted, centigrade	Hours barley extracted	Minutes between administering and vomition	Number of vomitions
2	20	15	room	24		0
1	100	15	room	24	12	9
2	100	15	room	24	24	15
1	100	15	7.2	48	16	5
2	300	32	7.2	168	70	8
15	240	27	92.0	12	19	9
16	240	27	92.0	12	19	4

The results tabulated in the above table indicate that the aqueous extracts contained the emetic principle. In only one case did an assay animal fail to respond and that was when the aqueous extract obtained from 20 grams of barley was administered. The data also indicate variation in the amount of emesis produced even when the aqueous extracts were of the same nature. Temperature and length of extraction are apparently not important factors. Amount and weight per bushel of the barley extracted seem to have some bearing on the toxicity of the aqueous extracts.

Effect of Adding Acid to an Aqueous Extract

The stability of the emetic principle toward acid was tested by adding hydrochloric acid to the aqueous extracts before administering to the assay animals. The results obtained from this treatment are given in the following table.

Table III

Effect of Acidifying an Aqueous Extract

Pig number	Grams of scabby barley extracted	Weight of barley per bushel, pounds	Temperature extracted, centigrade	Hours barley extracted	pH of aqueous extract	HCl added, pH	Minutes between administering and vomition	Number of vomitions
16	240	27	92	12	5.82	3.36	19	6
15	240	27	92	12	5.65	2.26	15	4

These results may be evaluated by comparing the number of vomitions of pigs number 15 and 16 in tables II and III. In all cases the barley was given the same treatment previous to making the two extracts more acidic. Apparently the addition of hydrochloric acid to an aqueous extract does not reduce the potency of the emetic principle.

Effect of Autoclaving an Aqueous Extract

In order to test the thermostability of the toxic substance, aqueous extracts were autoclaved at 15 pounds pressure for 25 minutes before their dispensation to swine. As indicated in table IV, the pH of the aqueous extracts ranged from 5.65 to 5.90.

Table IV

Effect of Autoclaving an Aqueous Extract at 15 Pounds Pressure for 25 Minutes

Pig number	Grams of scabby barley extracted	Weight of barley per bushel, pounds	Temperature extracted, centigrade	Hours barley extracted	pH of aqueous extract	Minutes between administering and vomition	Number of vomitions
1	100	15	7.2	48		28	4
16	240	27	92.0	12	5.90		*
15	240	27	92.0	12	5.65		0
16	240	27	92.0	12	5.74	22	1

* No emesis but appeared sick and refused feed.

The results indicate that autoclaving an aqueous extract reduces the potency of the emetic principle. Pig number 15 failed to react but pigs number 1 and 16 either vomited or appeared sick. A comparison of the reaction of pig number 16, when given an aqueous extract and an autoclaved aqueous extract, is possible from the results tabulated in tables II and IV. In each case 240 grams of 27 pound per bushel barley was given the same treatment previous to autoclaving. With the aqueous extract, however, more emesis was produced.

Effect of Autoclaving an Acidified Aqueous Extract

The reduction in potency of the toxic substance obtained from autoclaving an aqueous extract suggested autoclaving an acidified aqueous extract. Table V shows the results obtained from autoclaving the extracts after the latter had been adjusted to certain pH values by the addition of hydrochloric acid.

A comparison of the number of vomitions in tables IV and V substantiates the conclusion that the addition of hydrochloric acid to an aqueous extract before autoclaving had little if any effect. The data in both tables indicate a reduction in potency. Since the addition of hydrochloric acid before autoclaving did not reduce the potency of the emetic principle further than the autoclaving of an aqueous extract, it is doubtful if the acid has any effect.

Table V

Effect of Autoclaving an Acidified Aqueous Extract at 15 Pounds Pressure for 25 Minutes

Pig number	Grams of scabby barley extracted	Weight of barley per bushel, pounds	Temperature extracted, centigrade	Hours barley extracted	pH of aqueous extract	HCl added, pH	Minutes between administering and vomition	Number of vomitions
16	240	27	92	12	5.86	3.25	18	2
15	240	27	92	12	5.86	2.06		0
16	240	27	92	12	5.70	1.96		0
15	240	27	92	12	5.97	1.95	43	1
15	240	27	92	12	5.74	1.93	24	3
16	240	27	92	12	5.95	1.82		0
15	240	27	92	12	5.95	1.80		0

Effect of Adding Alkali to an Aqueous Extract

Beller and Wedemann (1929) have suggested that scabby barley may be utilized as feed for swine if the barley is extracted one hour with boiling water with subsequent washing or neutralization of the toxic substance with soda. Several attempts were therefore made to confirm this earlier work. The findings are reported in table VI. The pH values of the aqueous extracts are indicated and range from 5.56 to 5.94. Various amounts of NaOH solution were added to the extracts in order to obtain a succession

of pH values ranging from 7.15 to 11.65.

The data recorded in table VI show that a weakly basic solution of the aqueous extract is not effective in reducing the potency of the emetic principle. In only one case did a treated pig fail to show any symptoms. From these data it appears doubtful if an alkaline solution as strongly basic as indicated in table VI is effective in completely inactivating the toxic substance.

Table VI

Effect of Making an Aqueous Extract Alkaline

Pig number	Grams of scabby barley extracted	Weight of barley per bushel, pounds	Temperature extracted, centigrade	Hours barley extracted	pH of aqueous extract	NaOH added, pH	Minutes between administering and vomition	Number of vomitions
16	240	27	92	12	5.88	7.15	27	4
15	240	27	92	12	5.94	7.97	25	2
16	240	27	92	12	5.60	9.06	31	2
15	240	27	92	12	5.85	10.15	30	2
15	240	27	92	12	5.65	11.00		*
16	240	27	92	12	5.85	11.53	43	2
16	240	27	92	12	5.56	11.56		0
15	240	27	92	12	5.75	11.65		*

* No emesis but appeared sick and refused feed.

Effect of Evaporating an Aqueous Extract to Dryness

Thermostability data from table II suggested a possible means of concentrating the aqueous extract without altering the chemical nature of the emetic principle. The aqueous extract was divided equally among eight beakers and placed on a water bath for approximately four hours. As shown in table VII, the dried extracts were redissolved in water the same day they were obtained or allowed to remain as long as seven days in the dried state.

Table VII

Effect of Evaporating an Aqueous Extract to Dryness and Redissolving in Water Previous to Administering

Pig number	Grams of scabby barley extracted	Weight of barley per bushel, pounds	Temperature extracted, centigrade	Hours barley extracted	Days evaporated extracts remained in dried state	Minutes between administering and vomition	Number of vomitions
7	250	32	7.2	24	1	22	11
7	250	32	7.2	24	7	21	11
8	250	24	7.2	24	0	25	9
4	500	32	7.2	24	0	28	6
8	500	32	7.2	24	0	18	9

The data in the above table indicate that the redissolved dried extracts had not lost their ability to produce emesis in swine. The one

dried extract, which remained as such for seven days before redissolving and administering, was as effective in producing emesis as any dried extract tested.

Toxicity of the Filtered Concentrated Aqueous Extracts and Gummy Residues

During the removal of the water and other volatile substances from the aqueous extracts by evaporation over a water bath, a brown gummy residue formed on the sides of the beakers as well as in the concentrated extracts. In order to determine whether this material was toxic, the concentrated extracts were filtered with suction before being evaporated to dryness. Tests were made of the water soluble material and the gummy residue remaining on the filter paper and sides of the beakers. The results are summarized in table VIII.

Although pig number 3 vomited when administered the gummy residue, it is doubtful if this response is significant. As indicated, the gummy residue was not washed and due to the nature of the material it was easily possible for the emetic principle to remain behind. Further evidence, as indicated by the results from dispensing a gummy residue to pig number 4, suggests that this material is not toxic.

Vacuum, Steam and Fractional Distillation

Purification by vacuum distillation was attempted three times but, due to the foamy nature of the aqueous extracts when placed in a vacuum,

Table VIII

Toxicity of the Filtered Concentrated Aqueous Extracts and Gummy Residues

Pig number	Grams of scabby barley extracted	Weight of barley per bushel, pounds	Temperature extracted, centigrade	Hours barley extracted	Nature of material administered	Minutes between administering and vomition	Number of vomitions
2	800	32	7.2	216	Conc. extract	24	18
3	800	32	7.2	216	Gummy * residue	22	5
6	500	32	7.2	24	Conc. extract	17	7
4	500	32	7.2	24	Gummy** residue		0

* The gummy residue was not washed on the filter paper.
** The gummy residue was given a thorough washing with water.

Table IX

Toxicity of the Distillates and Residues Resulting from Steam Distillation

Pig number	Grams of scabby barley extracted	Weight of barley per bushel, pounds	Temperature extracted, centigrade	Hours barley extracted	Nature of material administered	Minutes between administering and vomition	Number of vomitions
3	100	15	7.2	52	Distillate		0
1	100	15	7.2	52	Residue	25	13
3	100	15	7.2	105	Residue	13	11
2	100	32	7.2	23	Residue	32	7

it was not possible to use this method.

The results obtained from subjecting concentrated aqueous extracts to steam distillation are presented in table IX.

The results in table IX show that the emetic principle remained in the residue. In the one case where the distillate was tested, the assay animal showed no symptoms of champing which generally precedes emesis. Pig number 3 continued eating throughout the one hour observed.

A further attempt to separate the emetic principle from the aqueous extract was made by concentrating the extract to a small volume and fractionally distilling. Table X gives the different fractions tested and the results obtained.

Table X

Toxicity of the Distillates and Residues Resulting from Fractional Distillation.

Pig number	Grams of scabby barley extracted	Weight of barley per bushel, pounds	Temperature extracted, centigrade	Hours barley extracted	Fraction administered, degrees centigrade	Minutes between administering and vomition	Number of vomitions
4	500	32	7.2	24	100-113		0
5					113-130		0
3					residue	18	9
5	500	32	7.2	24	100-130		0
3					130-170		0
4					residue	28	6

As in the case with steam distillation, the emetic principle remained in the residue. It seems quite significant that a distillation temperature of 170°C. did not inactivate the toxin.

Methyl and Ethyl Alcohols as Differential Solvents

After establishing the fact that the aqueous extracts could be taken to complete dryness (table VII) without a loss in the potency of the emetic principle, further methods were attempted to free the toxin from accompanying materials. The use of methyl and ethyl alcohols as differential solvents proved satisfactory in eliminating a large portion of the inactive material. To simplify the alcohol extractions, the aqueous extracts were removed from the water bath before evaporated to complete dryness and the alcohol added and warmed. It was necessary to use a spatula to free any residue adhering to the sides and bottoms of the beakers previous to mixing the alcohol with the concentrated material. The alcohol soluble material was filtered with suction and the insoluble material washed twice with alcohol. The data given in table XI reveal the efficiency of methyl alcohol as a solvent for the further purification of the toxic substance.

The results tabulated in table XI indicate that the methyl alcohol insoluble material is not toxic to swine. In one case, pig number 14, a response was obtained from dispensing the methyl alcohol insoluble material. This response may be explained by the fact that the insoluble material was not washed with methyl alcohol when filtered with suction.

Beller and Wedemann (1929) simplified their extraction method by placing the barley in boiling water for one hour. The evidence presented

Table XI

Toxicity of the Methyl Alcohol Soluble and Insoluble Portions Obtained from Extracting the Dried Residues Remaining After the Aqueous Extracts Were Evaporated to Nearly Dryness.

Pig number	Grams of scabby barley extracted with water	Weight of barley per bushel, pounds	Temperature extracted, centigrade	Hours barley extracted with water	Portion administered	Minutes between administering and vomition	Number of vomitions
9	250	24	7.2	24	soluble	25	9
10					insoluble		0
10	250	24	7.2	24	soluble	43	7
8					insoluble		0
12	160	24	7.2	24	soluble	14	4
11					insoluble		0
13	1200	27	7.2	12	insoluble		0
14	180	10	100.0	1	soluble	12	26
14	720	10	100.0	1	insoluble	32	4*
12	400	27	100.0	1	soluble	23	4

* Insoluble material not washed with methyl alcohol when filtered.

in table XI is the first instance of an attempt to confirm their recommendation. The results show that such a procedure does not inactivate the emetic principle. The amount of emesis from pig number 14 was greater than from any assay animal tested throughout the course of the investigation.

Ethyl alcohol proved to be a differential solvent, as indicated by the results summarized in table XII, but due to the fact that the insoluble portion had a tendency to remain in a gummy state, methyl alcohol was preferred for this step in the purification procedure.

Table XII

Toxicity of the Ethyl Alcohol Soluble and Insoluble Portions Obtained from Extracting the Dried Residue Remaining After the Aqueous Extract Was Evaporated to Dryness

Pig number	Grams of scabby barley extracted with water	Weight of barley per bushel, pounds	Temperature extracted, centigrade	Hours barley extracted with water	Portion administered	Minutes between administering and vomition	Number of vomitions
11	250	24	7.2	24	soluble	10	16
12					insoluble		0

Ether as a Differential Solvent

In order to eliminate any foreign material soluble in both water and methyl alcohol, ether was employed as a further differential solvent to extract the methyl alcohol soluble material. Aqueous solutions of the methyl alcohol soluble material were extracted with a separatory funnel or the continuous extractor shown in figure 1. The results from several trials are given in table XIII.

The results tabulated in this table show that the emetic principle

Table XIII

Toxicity of the Ether Soluble and Insoluble Portions Obtained from Extracting the Concentrated Methyl Alcohol Soluble Material

Pig number	Grams of scabby barley* extracted with water	Method of ether extraction	Number of ether extractions	Hours of ether extraction	Portion administered	Weight of ether soluble material, grams	Minutes between administering and vomition	Number of vomitions
11	250	separatory funnel	10		soluble		14	5
12					insoluble			0
12	300	separatory funnel	10		soluble		16	9
11					insoluble		19	11
11	300	separatory funnel	15		insoluble		22	9
11	300	continuous		12	soluble	0.2740	15	6
12					insoluble		21	3
12	300	continuous		24	insoluble			0
12	300	continuous		36	insoluble			0
14	180	continuous		24	soluble	0.5370	14	18
14					insoluble			0

* Barley of various bushel weights extracted with water at 92°C. for 12 hours.

was present in the ether soluble and insoluble portions. The separatory funnel did not appear to be suitable for completely removing the toxin from the aqueous portion. The results obtained from using the continuous extractor were more promising. Twelve hours continuous extraction was not sufficient to completely remove the emetic principle, but extractions of 24 and 36 hours proved satisfactory. After evaporating the ether off, only a small amount of ether soluble material remained and this was a yellow syrupy substance. Table XIII indicates the weights of this material on two occasions. As little as 0.5370 grams was sufficient to make assay animal number 14 vomit 18 times.

Benzene as a Differential Solvent

The physical properties of benzene suggested the possibility of using this solvent for continuous extraction from an aqueous solution of the methyl alcohol soluble material. The results from one trial are given in table XIV.

The evidence obtained from one extraction indicates that benzene is not a suitable solvent for further purification of the emetic principle.

Ether Extractions from Basic and Acidic Aqueous Solutions

The promising results given in table XIII suggested further methods for purification as well as an opportunity to learn something of the chemical nature of the toxic substance. The acid reaction of every aqueous extract, the inactivation of the toxin with sodium hydroxide as reported

Table XIV

Toxicity of the Benzene Soluble and Insoluble Portions Obtained from Extracting the Concentrated Methyl Alcohol Soluble Material.

Pig number	Grams of scabby barley* originally extracted with water	Hours of continuous extraction with benzene	Portion administered	Minutes between administering and vomition	Number of vomitions
12	300	36	soluble		0
13			insoluble	16	7

* Barley weighing 27 pounds per bushel extracted with water at 92°C. for 12 hours.

by Beller and Wedemann (1929), but not confirmed in this investigation, and the solubility of the concentrated methyl alcohol soluble material in dilute sodium hydroxide solution all suggested that the unknown might have one or more acidic groups strong enough to react with sodium hydroxide and thus be insoluble in ether. An opportunity to determine whether the unknown would form an ether insoluble hydrochloride salt was presented by attempting to extract an acidified aqueous solution of the concentrated methyl alcohol soluble material with ether. Data obtained from several extractions are summarized in table XV.

The results shown in table XV indicate that the emetic principle may be extracted from either a basic or acidic aqueous solution. Evidently the unknown is not strongly acidic nor has the property of forming a hydrochloride salt. One significant comparison is the amount of ether

Toxicity of the Ether Soluble and Insoluble Portions Obtained from Extracting Basic and Acidic Solutions.*

Pig number	Acid or base added	pH of solution extracted from	Times extracted with ether	Hours of continuous ether extraction	Portion administered	Weight of ether soluble portion	Minutes between administering and emesis	Number of vomitions
12	5 ml. HCl**		15		soluble		15	7
11					insoluble			0
11	5 ml. NaOH***		15		soluble		23	6
12					insoluble			0
12	5 ml. HCl			36	soluble	0.7200	18	8
11					insoluble			0
12	10 ml. NaOH			12	soluble	0.0595		0
13					insoluble			0
14	NaOH	8.67		24	soluble	0.1730	9	18
14	HCl	0.45		24	soluble	0.7500	25	8
14	NaOH	8.60		24	soluble	0.1500	26	3
15	NaOH	8.40		24	soluble	0.1140	16	2
16	NaOH	11.35		24	soluble	0.0770	50	3
15	NaOH	9.90		24	soluble	0.1250	33	2

* Original aqueous extracts made from barley of various bushel weights at 92°C. for 12 hours.
** Concentrated HCl.
*** Forty percent NaOH

soluble material obtained from the basic and acidic extractions. From two acid extractions 0.7200 and 0.7500 grams of the yellow syrupy substance were obtained. The largest amount of ether soluble material obtained from a basic extraction was 0.1730 grams. This reduced amount of ether soluble material was still as potent in affecting emesis as the larger amount obtained from the acidic extractions. In one case, assay animal number 16 vomited three times on 0.0770 grams of the yellow syrupy substance. Ether extractions from basic solutions tend to eliminate approximately 0.5 grams of foreign material which ordinarily was included in the ether soluble material extracted from an acidic solution.

Toxicity of Methyl and Ethyl Alcohols

Due to the fact that methyl and ethyl alcohols were used as differential solvents, it is entirely possible that traces of these alcohols remained in some of the test solutions given to the assay animals. Weak solutions were therefore administered to assay animal number 14 to determine whether emesis was produced.

Beller and Wedemann (1929) administered one, three, five and seven percent alcoholic solutions to swine but no reaction was evident in any case. The results tabulated in table XVI confirm this earlier work. It is most certain that traces of methyl or ethyl alcohol had no effect because five and ten percent solutions are many times stronger than could have possibly remained in the yellow syrupy material.

Table XVI

Toxicity of Methyl and Ethyl Alcohols

Pig number	Alcohol	Milliliters of solution given	Strength of solution, percent	Apparent effect
14*	methyl	100	5	none
14	ethyl	100	10	none

* Pig number 14 was very susceptible to the emetic principle.

Attempts to Precipitate and Crystallize

Two attempts were made to precipitate the emetic principle as a hydrochloride salt by bubbling dry hydrogen chloride gas through an ethereal solution of the toxin which had previously been dried with anhydrous sodium sulphate. In both cases, a dark yellow, oily appearing substance came down.

In an attempt to crystallize the emetic principle from ethyl alcohol by the addition of water, a flocculent, tan colored precipitate appeared. Upon filtration, this precipitate appeared similar to the original syrupy material.

Further attempts to crystallize the yellow syrupy substance were made by placing some at -28.8°C. for 10 days. Upon removal, the toxin appeared exactly as it did before placing it in the refrigerator. Twenty weeks in a desiccator also failed to bring about a change in the syrupy material.

Even after that length of time, little if any hardening of the toxin was noticeable.

Physical Examination

The emetic principle was collected by pouring the ether soluble material on a watch glass. Subsequent to the evaporation of the ether, the toxin appeared as a homogeneous, yellow syrupy material which would run slowly when the watch glass was inclined. The yellow material thickened somewhat on prolonged standing in a desiccator but failed to harden after standing as long as 20 weeks.

The yellow syrupy material had an unfamiliar odor. Prolonged smelling produced headache in man. A slightly bitter taste was characteristic of the concentrated material.

An examination of the combustibility of the yellow syrupy material indicated that it burned slowly with a red flame. Carbon, but no inorganic residue, remained.

Reaction of the Yellow Syrupy Concentrate

Reference to tables III, IV, V and VI indicates that the aqueous extracts from the whole grain were always acidic. Readings of the alcoholic solutions of the syrupy material are given in table XVII. The yellow syrupy material was obtained by extracting concentrated aqueous solutions of the emetic principle with ether.

The pH readings of the yellow syrupy material are more acidic than

the original aqueous extracts. A negative correlation appears to exist between the weight of the toxin and the pH reading.

Table XVII

pH Readings of the Yellow Syrupy Material

Grams	pH
0.0885	3.16
0.1980	3.03
0.5370	2.65

Elementary Analysis

Analysis for sulfur, nitrogen, chlorine, bromine and iodine was made by using the sodium fusion method outlined by Kamm (1932). The analysis for sulfur and the halogens was negative but a positive test was obtained for nitrogen.

Solubility Tests

Although water and ether were used as solvents for the extraction and concentration of the emetic principle, the yellow syrupy material obtained was very difficultly soluble in water and difficultly soluble in ether. Solvents in which the concentrated material was insoluble were dilute hydrochloric acid, dilute sodium bicarbonate, benzene, petroleum ether, carbon tetrachloride and carbon disulphide. Solvents in which the material was miscible were methyl alcohol, ethyl alcohol, dilute sodium hydroxide,

acetone, chloroform and 1,4-dioxane.

According to the scheme outlined by Shriner and Fuson (1935), the solubility behavior would indicate an acidic compound falling in the solubility class A_2. This class includes phenols, sulfonamides of primary amines, primary and secondary nitro compounds, imides and thiophenols. Since the yellow syrupy material may not be a pure substance, the solubility observations are of questionable value.

Homologous Tests

The ferric chloride test was used to test for the presence of a phenolic type of compound. Addition of ferric chloride solution to a yellow, alcoholic solution of the concentrate turned the latter to a darker yellow. When ferric chloride solution was added to an aqueous solution of the concentrate no color change occurred.

To test for the presence of a nitro compound, sodium hydroxide solution was added to an alcoholic solution of the concentrate. A change occurred from the normal yellow color to a reddish yellow.

In addition to the above homologous tests, the solubility behavior of the unknown concentrate adds further information concerning its nature. Immiscibility in benzene suggests the elimination of a phenolic type of compound as well as an aromatic nitro compound.

Acetyl chloride was used to test for the presence of an acidic hydrogen but no reaction occurred. Negative results were also obtained when using bromine to test for the presence of unsaturation.

The yellow color of the concentrated material suggested the possibility

of one or more nitro groups. After one attempt at reduction in the presence of tin and hydrochloric acid over a steam bath for 20 minutes, the yellow syrupy material was recovered and appeared the same and had the characteristic odor. It was not tested for toxicity.

The inference obtained from the above homologous tests is not conclusive enough to draw any definite conclusions. For this reason a number of derivatives were attempted, regardless of the outcome of the above tests, in order to learn more concerning the chemical behavior of the unknown as well as to attempt to isolate it as a derivative.

Attempted Derivatives

The acid reaction of the yellow syrupy material, as indicated in table XVII, suggested the unknown might form an ester, even though the solubility data indicated the toxin was not an organic acid. The procedures outlined by Shriner and Fuson (1935) were followed in attempting to form the p-nitrobenzyl and p-bromophenacyl esters. The p-nitrobenzyl bromide and p-bromophenacyl bromide were recovered indicating no reactions had taken place.

To support previous evidence that the unknown might be a phenolic type of compound, attempts were made to brominate it and to form a diphenylurethan by using diphenylcarbamyl chloride. The procedures given by Shriner and Fuson (1935) were followed in the preparation of these derivatives. In both cases, the results were negative.

Since the yellow syrupy material gave a positive test for nitrogen, the inference was made that it might be a tertiary amine or alkaloid. Methyl iodide was used, in accordance with the procedure of Shriner and

Fuson, in an attempt to form a quaternary ammonium salt but no precipitate formed.

The procedure for the formation of picrates is relatively simple and such derivatives are not restricted to one class of compounds. For the latter reason, the concentrate was treated with picric acid, according to the procedure given by Shriner and Fuson (1935), but no reaction took place.

ALKALOIDAL TESTS

The yellow syrupy material was tested with Marquis', Mayer's, Hager's and Scheibler's reagents in order to detect the presence of an alkaloid. Marquis' reagent gave a wine-red coloration which turned brown upon the addition of more of the reagent. Mayer's, Hager's and Scheibler's reagents did not give a precipitate typical of a positive test for alkaloids. In each case, these reagents produced a cloudiness.

Since the above tests could not be considered as being positive, an attempt was made to purify the material further and test for alkaloids again. The yellow syrupy material was dissolved in a mixture of chloroform and ether and extracted with a two percent hydrochloric acid solution. The acid soluble portion was then tested with various alkaloidal reagents. This portion gave positive tests with Mayer's reagent, gold chloride, iodine, neutral lead acetate and phosphotungstic acid. A negative test was obtained when using platinum chloride. These tests indicated that the acid soluble portion contains complex nitrogen bases.

DISCUSSION

Various attempts to inactivate the toxin are sufficient to show that it is quite stable toward heat and to changes in the hydrogen ion concentration of the medium (water) within which it is dissolved. The procedure used to concentrate the toxic material indicates further that it is not destroyed when the impure mixture within which it is contained is taken to complete dryness. It is possible that the toxin is altered by these various treatments but an active group may remain and cause emesis.

Solubility data obtained during the concentration of the emetic principle were helpful but still somewhat confusing. For instance, table XV shows that the toxin may be extracted from a basic or acidic aqueous solution with ether. One would not expect organic compounds which were acidic enough to form a sodium salt with sodium hydroxide to be extracted in the former case or for a complex nitrogen base to be extracted in the latter case. The solubility behavior of the yellow syrupy concentrate indicates an acidic compound. Following the scheme outlined by Shriner and Fuson (1935), the unknown would fall into the solubility class A_2. This class includes phenols, sulfonamides of primary amines, primary and secondary nitro compounds, imides and thiophenols. Homologous tests, attempted derivatives and the elementary analysis tend to eliminate these types of compounds.

The fact must be borne in mind that the yellow syrupy concentrate may not be pure and not being pure could interfere with the various tests

used to identify the unknown. Possibly the concentrated material is not pure, but the fact still remains that it was very potent when in the form of the yellow syrupy material. Table XV shows that as little as 70 milligrams was potent enough to cause pig number 18 to vomit three times.

The elementary analysis indicated the presence of nitrogen. It is of value to know this element is present even though the yellow syrupy material may not be pure. If the nitrogen test were negative, it would eliminate the possibility that the emetic principle was an alkaloid.

The positive response to various alkaloidal reagents suggests that one or more complex nitrogen bases are present in the yellow syrupy material. Since only a portion of this syrupy material is soluble in dilute hydrochloric acid when extracted from a chloroform-ether mixture, it appears as though the purification is not complete. If this be the case and all evidence points to that conclusion, it is doubtful if the solubility tests on the yellow syrupy material are of much significance.

SUMMARY

1. The emetic principle present in barley infected with Gibberella saubinetii is very stable toward heat.

2. Changes in the hydrogen ion concentration of the medium (water) in which the emetic principle is dissolved do not inactivate it.

3. In case the toxin is altered by various treatments used to inactivate it, an active group may remain which causes emesis.

4. Original aqueous extracts can be concentrated to a very small volume and still retain the potency of the former.

5. The concentrated extracts appeared as a yellow syrupy material. As little as 70 milligrams of this syrupy material produced emesis in swine.

6. Since the evidence points to the fact that this yellow syrupy concentrate is not pure, it is doubtful if its solubility behavior is of much significance.

7. The yellow syrupy concentrate gave a positive test for nitrogen. A negative test would have eliminated the possibility that the emetic principle was an alkaloid.

8. Further purification of the yellow syrupy concentrate was obtained by dissolving it in a chloroform-ether mixture and extracting with dilute hydrochloric acid. The acid soluble portion responded to various alkaloidal reagents such as Mayer's reagent, gold chloride, iodine, neutral lead acetate and phosphotungstic acid.

9. The toxicity of the acid soluble portion which gave a positive test with the various alkaloidal reagents has not been demonstrated.

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